EXHIBIT 1

ELSEVIER

Inhibitors of the tyrosine kinase signaling cascade for asthma

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The pathogenesis of allergic asthma involves the interplay of inflammatory cells and resident airway cells, and of their secreted mediators including cytokines, chemokines, growth factors and inflammatory mediators. Tyrosine kinase signaling cascades play a critical role in the pathogenesis of allergic airway inflammation. Receptor tyrosine kinases (e.g. epidermal growth factor receptor [EGFR] and platelet-derived growth factor receptor) are important for the pathogenesis of airway remodeling. Stimulation of non-receptor tyrosine kinases (e.g. Lyn, Lck, Syk, ZAP-70, Btk, Itk and JAK) is the earliest detectable signaling response upon activation of immune receptors (T cell receptor, B cell receptor and FCεR1), cytokine receptors and chemokine receptors in inflammatory cells. Activation of tyrosine kinases invokes multiple downstream signaling pathways, including phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB), leading to cell differentiation, survival, proliferation, degranulation and chemotaxis. Inhibitors targeted at different enzyme molecules of the tyrosine kinase signaling cascade might afford therapeutic potential for asthma. Anti-inflammatory effects of pharmacological agents targeted at tyrosine kinases, Syk, Itk, signal transducer and activator of transcription-1, NF-κB, GATA3, EGFR, PI3K, MEK1/2, p38 MAPK and JNK have been reported in animal models of allergic airway inflammation. Therefore, development of inhibitors targeted at the tyrosine kinase signaling cascade is an attractive strategy for the treatment of asthma.

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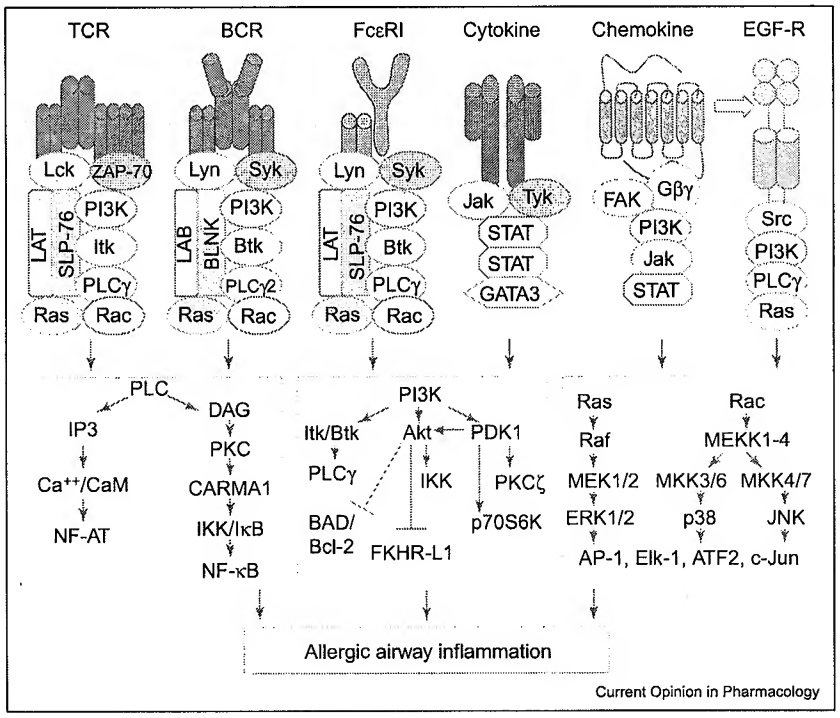
Introduction

Asthma is a chronic disease of the lungs characterized by bronchial eosinophilic inflammation and airway hyperresponsiveness. Lung biopsy studies in asthmatics have revealed pulmonary infiltration of eosinophils, lymphocytes, macrophages and mast cells, and structural changes of the airways including airway wall thickening, subepithelial fibrosis, and goblet cell and airway smooth muscle cell hyperplasia that are commonly referred to as airway remodeling [1,2]. T-helper 2 (Th2) cells together with other inflammatory cells and resident airway cells are proposed to play a critical role in the initiation and perpetuation of allergic airway inflammation [3]. Interleukin (IL)-4, IL-5 and IL-13 are the best-characterized Th2 cytokines. IL-4 is important for Th2 response development and promotes B cell switching to IgE production. IL-13 promotes mucus production, airway hyperresponsiveness, eotaxin expression and transforming growth factor-β production [4]. IL-5 is important for eosinophil terminal differentiation, recruitment, activation and airway remodeling [5]. Crossing-linking of IgE-bound FcεRI on mast cells leads to degranulation and immediate release of histamine, leukotrienes, tryptase and other pro-inflammatory mediators. Mast cell-derived tryptase has been shown to promote airway eosinophilia, hyperplasia, fibrosis and hyperresponsiveness via activation of the proteinase-activated receptor 2, a member of the seven-transmembrane G-protein-coupled receptor (GPCR) superfamily [6]. Chemokines such as eotaxin and RANTES (regulated on activation normal T cell expressed and secreted) play a critical role in recruiting T cells and eosinophils to the airways. Chemokine receptors constitute another subfamily of GPCRs. Both Th2 cells and eosinophils express high levels of CCR3, which can be selectively activated by eotaxin, RANTES and several other chemokines [7]. It has been shown that expression of epidermal growth factor (EGF) and its receptor (EGFR) is increased in asthmatic human airways [8]. In human airway smooth muscle cells, both EGF and platelet-derived growth factor have been shown to promote EGFR and platelet-derived growth factor receptor tyrosine autophosphorylation, leading to transcription factor activation and proliferation [9]. EGFR activation also plays a major role in modulating mucus production in airway epithelium. Asthma is associated with airway goblet cell hyperplasia and enhanced mucin gene expression. EGF and eosinophil-derived transforming growth factor-α, another natural ligand for the EGFR, have been shown to stimulate MUC5AC mucin gene expression and protein synthesis in a human airway epithelial cell line, and goblet cell proliferation [10]. Cumulative findings indicate that protein tyrosine kinase signaling cascades are closely involved in almost all processess of the pathogenesis of asthma, and that pharmacological inhibition of some of these critical signaling molecules might have therapeutic effects for asthma (Figure 1) [11].

Figure 1



Tyrosine kinase signaling cascades activated by various transmembrane receptors leading to the pathogenesis of allergic airway inflammation. AP-1, activator protein 1; BCR, B cell receptor; CaM, calmodulin; DAG, diacylglycerol; FAK, focal adhesion kinase; FcεRI, high-affinity IgE-binding receptor; FKHR-L1, forkhead related-ligand 1; IP_3, inositol (3,4,5) triphosphate; IκB, inhibitory κB; IκK, IκB kinase; Itk, inducible T cell kinase; LAB, linker for activation of B cells; p70S6K, p70S6 kinase; PDK1, phosphoinositide-dependent kinase 1; NF-AT, nuclear factor of activated T cells; SLP-76, SH2 domain-containing leukocyte protein of 76 KDa; Syk, spleen tyrosine kinase.

This review begins with discussion of the role of tyrosine kinase signaling cascades in mediating activation of T cell receptors (TCRs), B cell receptors, FcεRI, cytokine receptors, GPCRs and receptor tyrosine kinases, as well as their role in inflammatory responses. This is followed by a summary of the effects of inhibitors of the tyrosine kinase signaling cascades in various animal models of allergic asthma. The review concludes by discussing the potential use of inhibitors targeted at the tyrosine kinase signaling cascade for the treatment of asthma.

Tyrosine kinase signaling cascades in asthma

Receptor tyrosine kinases

Protein tyrosine kinases (both receptor tyrosine kinases and non-receptor tyrosine kinases) are essential for the activation and proliferation of inflammatory cells and resident airway cells. Growth factor receptors (e.g. EGFR and platelet-derived growth factor receptor) are transmembrane receptor tyrosine kinases. Upon activation, these receptors undergo dimerization and tyrosine autophosphorylation, followed by recruitment and activation of signaling molecules that contain src homology 2 (SH2) and phosphotyrosine binding domains, including non-receptor tyrosine kinase Src, phosphoinositide 3-kinase (PI3K) and phospholipase Cγ (PLCγ), resulting in airway smooth muscle proliferation and mucus hypersecretion [9,10].

Non-receptor tyrosine kinases

The earliest detectable signaling response upon immunoreceptor activation in mast cells [12], T cells [13] and B cells [14] is the stimulation of non-receptor tyrosine kinases. Immune receptors, including the high-affinity IgE receptor (FcεRI), TCR and B cell receptor, are composed of antigen-binding subunits and signal transducing subunits. The signal transducing chain is encoded with one or more copies of an unique amino acid sequence [Y(2X)I/L(6–8X)Y(2X)I/L] termed immunoreceptor tyrosine-based activation motifs (ITAMs) [15]. Upon tyrosine phosphorylaton activation, ITAM serves as a docking site for downstream signaling molecules and adaptor proteins containing SH2 or phosphotyrosine binding domains, leading to multiple cascades of signal transmission. For TCR activation, ITAMs located in the CD3 molecule are tyrosine phosphorylated by Lck and Fyn, two Src family tyrosine kinases, followed by recruitment and activation of SH2-containing ZAP-70, a mem-

ber of the Syk family tyrosine kinase. These activated tyrosine kinases then phosphorylate downstream adaptor molecules such as LAT (linker for activation of T cells) and SLP-76 (SH2 domain-containing leukocyte protein of 76 kDa), leading to activation of multiple downstream signaling molecules such as inducible T cell kinase (Itk; a member of the Tec family tyrosine kinase), PLCγ1 and PI3K [13]. For B cell receptor activation, ITAMs are tyrosine phosphorylated by Src-family kinases such as Lyn, Fyn or Blk, followed by Syk recruitment and activation. Tyrosine phosphorylation of BLNK (also known as SLP-65) and linker for activation of B cells (LAB) by Syk leads to activation of Bruton's tyrosine kinase (Btk; another member of Tec family tyrosine kinases), PLCγ2 and PI3K for downstream signal propagation [14]. FcεRI is a tetrameric protein ($\alpha\beta\gamma_2$ chains) consisting of the IgE-binding α chain, a signal-amplifying β chain and two disfulfide-linked γ chains, which contain the ITAMs for signal transduction. Cross-linking of FcεRI by multivalent antigen activates Lyn, a member of Src-family kinases, followed by Syk recruitment and activation. This is followed by tyrosine phosphorylation of LAT and SLP-76 and activation of Btk, PLCγ and PI3K, leading ultimately to mast cell degranulation and activation [12]. Tec family kinases can activate PLCγ leading to hydrolysis of phophatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] into inositol (3,4,5)-trisphosphate and diacylglycerol, which in turn mobilize intracellular Ca^{2+} and activate isoforms of protein kinase C (e.g. PKCβ, PKCζ and PKCθ) [16]. Isoforms of PKC are essential for activation of the transcription factor nuclear factor-κB (NK-κB) by immune receptors via sequential phsophorylation and stimulation of CARMA1/BCL10/MALT1 and inhibitory κB kinases, and ubiquitination of inhibitory κB kinases [17]. Increased NK-κB activity in lung cells has been observed in asthmatic subjects and in animal models of asthma, and is essential for Th2 differentiation in allergic airway inflammation [18,19].

PI3K pathway

Upstream tyrosine kinase activation and phosphorylation of ITAM and adaptor proteins generate multiple downstream signaling cascades of which the PI3K and mitogen-activated proein kinase (MAPK) pathways are considered critical for inflammatory cell activation, differentiation, survival, chemotaxis and proliferation. PI3Ks are divided into four classes, namely IA, IB, II and III, on the basis of their structural characteristics and substrate specificities. Class IA PI3K is activated by tyrosine phosphorylation and its genetic knockout mice reveal impaired T cell, B cell and mast cell responses [20,21]. Upon activation, PI3K converts the plasma membrane lipid PtdIns(4)P and PtdIns(4,5)P_2 to PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, respectively, by phosphorylation of the D3 position of the inositol ring of the phospholipids. PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 then act as targeting sites for downstream signaling effectors with pleckstrin homology, phox homology or FYVE domains, including serine/threonine kinases of the AGC (cAMP- and cGMP-dependent protein kinases and protein kinase C) family, such as Akt (also called protein kinase B) and phosphoinositide-dependent kinase 1, and the Tec family of tyrosine kinases, such as Tec, Btk and Itk [22]. As mentioned above, Tec kinase can activate PLCγ leading to mobilization of intracellular Ca^{2+} and activation of PKC isoforms [16]. Class 1B PI3K is stimulated by chemokine GPCR activation via the Gβγ subunit of the heterotrimeric G protein, and its genetic knockout mice reveal defective dendritic cell migration in response to chemokines and impaired adaptive immunity [23].

MAPK pathway

MAPK signaling cascades can be initiated upon activation of immune receptors, growth factor receptors, cytokine receptors and chemokine receptors through a three-tiered sequential phosphorylation of MAPK kinase kinase (MAP3K or MKKK), MAPK kinase (MAP2K or MEK or MKK) and MAPK. There are three major groups of MAPK in mammalian cells: extracellular signal-regulated protein kinase (ERK), p38 MAPK and c-Jun NH_2-terminal kinase (JNK). ERK1 and ERK2 are activated by dual phosphorylation at the tripeptide motif Thr-Glu-Tyr by their direct upstream kinases MEK1 and MEK2, respectively. p38 MAPKs exist in four distinct isoforms (α, β, γ and δ) and are activated by dual phosphorylation at a Thr-Gly-Tyr motif by their direct upstream kinases MKK3 and MKK6. JNK1, 2 and 3 are activated by dual phosphorylation at a Thr-Pro-Tyr motif by their direct upstream kinases MKK4 and MKK7 [24]. The phosphorylation states and/or activities of all three MAPK members (ERK, p38 MAPK and JNK) are upregulated in animal models of asthma [25,26,27••]. In turn, MAPK enhances the transcriptional activities of activation protein 1, ATF2 and c-Jun, leading to gene expression, cytokine production, differentiation and proliferation of inflammatory cells [24].

JAK-STAT pathway

Cytokine receptors are transmembrane receptors and can be classified into five families: gp130 family, IL-2 receptor (γ chain) family, growth hormone (single-chain) family, IFN family and gp140 family. Receptors for IL-4 and IL-13 belong to the γ chain family, which uses a common γ subunit for signal transduction. By contrast, IL-5 receptor uses the gp140 family (β chain) for signal transduction. Cytokines induce receptor oligomerization and activation of a family of non-receptor tyrosine kinase, the Janus kinases (JAK1, JAK2, JAK3 and TYK2). Each JAK isoform is specifically linked to the cytoplasmic tails of different cytokine receptors, leading to tyrosine phosphorylation of particular cytokine receptors and subsequent recruitment and activation of a family of transcription factors named STAT (signal transducer and activator of transcription). STAT has seven family

members and phosphorylated STATs form homo- or hetero-dimers in the cytoplasm and subsequently translocate to the nucleus to regulate selective gene expression [28]. Activation of IL-4 receptor stimulates JAK1 (associated with IL-4Rα chain) and JAK3 (associated with γ chain) followed by selective activation of STAT6. Conversely, IL-13 can activate both IL-4Rα chain and IL-13Rα1 chain to stimulate JAK1 and TYK2, leading to selective activation of STAT6. STAT6 is critical for Th2 cell differentiation through selective upregulation of a Th2-selective transcription factor GATA3. STAT6-deficient mice failed to develop allergic airway inflammation [29]. Genetic ablation of GATA3 suppressed Th2 cell differentiation and proliferation, and Th2 cytokine expression [30].

GPCR pathway

Activation of chemokine receptors triggers multiple cascades of signaling events that lead to recruitment and activation of immune effector cells. There is increasing evidence showing that chemokines induce chemokine receptor dimerization and JAK-STAT signaling pathway activation. RANTES invokes rapid tyrosine phosphorylation of its cognate receptor CCR5 on human T cells, activation of JAK2 and JAK3 and formation of STAT1 and STAT3 dimers, stimulation of PI3K activity via the Gβγ subunit, and activation of ZAP-70 tyrosine kinase and focal adhesion kinase via transactivation of the TCR [31]. GPCR agonists such as thrombin, bradykinin and lysophosphatidic acid can also invoke tyrosine kinase signaling cascades via transactivation of receptor tyrosine kinases (e.g. EGFR), leading to PI3K and MAPK pathway stimulation in airway smooth muscle cells [32].

The role of tyrosine kinase signaling cascades in allergic airway inflammation is beginning to emerge and, as these cascades are triggered by a variety of immune-associated receptor systems, inhibition of these signaling cascades is expected to produce anti-inflammatory and anti-remodeling effects in asthma [33••].

Inhibitors of the tyrosine kinase signaling cascade for asthma

Tyrosine kinase inhibitors

For an extensive review on tyrosine kinase inhibitors in asthma, please read a recent review by Wong and Leong [33••]. A recent study using an isoflavone compound, genistein (a broad-spectrum non-selective tyrosine kinase inhibitor), showed that this significantly attenuated antigen-induced acute bronchoconstriction and airway hyperresponsiveness to inhaled methacholine in an *in vivo* guinea-pig model of asthma. Genistein also reduced antigen-induced increases in total cell count, eosinophil count and eosinophil peroxidase activity recovered in bronchoalveolar lavage (BAL) fluid, and airway eosinophilia (Table 1) [34••]. The non-receptor tyrosine kinase Syk is critically involved in immunoreceptor signaling in leukocytes, and selective Syk inhibition might be beneficial for asthma [35•]. Using aerosolized Syk-selective antisense oligonucleotides (ASOs), Stenton *et al.* [36] showed that antigen-induced eosinophil infiltration into the lungs, leukocyte cell surface expression of adhesion molecules such as β_2 integrin, α_4 integrin and intercellular adhesion molecule-1, and tumor necrosis factor-α (TNF-α) production were suppressed in a rat model of asthma. In addition, an orally available selective Syk inhibitor (BAY61-3606) dose-dependently suppressed antigen-induced acute bronchoconstriction and bronchial edema in a passively sensitized rat model of acute asthma, as well as antigen-induced airway esoinophilia in an actively sensitized rat model of asthma [37]. Itk, a T cell-specific member of the Tec family of tyrosine kinases, plays a key role in relaying upstream TCR activation signals to downstream PLCγ1 activation and Ca^{2+} mobilization [16]. Mice lacking Itk have drastically reduced allergic airway inflammation [38]. Itk-selective inhibitors BMS-488516 and BMC-509744 have recently been shown to suppress the production of IL-2 induced by anti-TCR antibody when given to mice. In addition, BMC-509744 dose-dependently prevented antigen-induced leukocyte infiltration into the lung in a mouse model of asthma [39••]. JAK3 is important for common γ chain-associated cytokine receptor signaling, which drives IL-4-mediated Th2 responses and STAT6 activation. The JAK3 inhibitor WHI-P97 has been shown to prevent antigen-induced airway hyperresponsiveness and eosinophil infiltration into the airways. Nevertheless, the specificity of WHI-P97 for JAK3 remains to be determined [40]. CP-690560 is by far the most selective inhibitor for JAK3 being developed and has been shown to be a potent immunosuppressant in a transplantation model [41]. As JAK3 is critical for IL-4-mediated Th2 development, CP-690560 is anticipated to demonstrate a beneficial effect in asthma. STAT1 activity is upregulated in epithelial cells of asthmatic patients, which is critical for the expression of genes encoding co-stimulatory molecules (e.g. CD40) and adhesion molecules (e.g. vascular cell adhesion molecule [VCAM-1]). Using intratracheally administered STAT1-selective decoy oligodeoxynucleotides, Guarcoo *et al.* [42••] have recently demonstrated anti-inflammatory effects on antigen-induced airway eosinophil infiltration, cytokine production, hyperresponsiveness and expression of CD40 and VCAM-1 in a mouse model of asthma. NF-κB is a critical transcription factor for Th2 cell differentiation and cytokine production in allergic airway inflammation [17–19]. Intratracheal administration of decoy oligodeoxynucleotides targeted at NF-κB strongly attenuated allergic airway inflammation, airway hyperresponsiveness, and local production of mucus, IL-5, IL-13 and eotaxin in a mosue asthma model [43••]. In a separate study using ASOs targeted at NF-κB, airway inflammatory cell infiltration, local IL-4, IL-5 and TNF-α production, serum levels of

Table 1

Effects of inhibitors of the tyrosine kinase signaling cascades in animal models of asthma.

Target	Inhibitor	Model	Eosinophilia	Mucus	BAL fluid cytokine	Serum IgE	AHR	Others	Ref
Tyrosine kinases	Genistein	Guinea- pig	↓	ND	ND	ND	↓	↓ EPO activity in BAL and acute bronchoconstriction	[34]
Syk	ASO	Rat	↓	ND	↓ TNF-α	ND	ND		[36]
	BAY61-3606	Rat	↓	ND	ND	ND	ND	↓ bronchial edema and acute bronchoconstriction	[37]
Itk	BMC-509744	Mouse	↓	ND	ND	ND	ND		[39]
JAK3	WHI-P97	Mouse	↓	ND	ND	ND	↓		[40]
STAT1	Decoy ODN	Mouse	↓	ND	↓ IL-5; ↔ IL-4	ND	↓	↓ CD40 and VCAM-1 in lung tissues	[42]
NF-κB	Decoy ODN	Mouse	↓	↓	↓ IL-5, IL-13, eotaxin; ↔ IL-4	↔	↓		[43]
	ASO	Mouse	↓	ND	↓ IL-4, IL-5, TNF-α	↓	↓		[44]
GATA3	ASO	Mouse	↓	↓	↓ IL-4	ND	↓		[45]
EGFR	AG-1478	Mouse	↓	↓	ND	ND	↓	↓ MUC5AC in BAL fluid and ↓ collagen deposition	[46]
PI3K	LY294002 DN PI3K-TAT	Mouse	↓	↓	↓ IL-4, IL-5, IL-13, eotaxin	ND	↓	↓ ECP in BAL fluid	[47–49]
ERK	U0126	Mouse	↓	↓	↓ IL-4, IL-5, IL-13, eotaxin	↓	↓	↓ VCAM-1 in lung tissues	[50]
P38 MAPK	SB239063	Guinea- pig Mouse	↓	ND	ND	ND	ND		[51]
	ASO	Mouse	↓	↓	↓ IL-4, IL-5, IL-13	ND	↓		[52]
JNK	SP600125	Rat	↓	ND	↔ IL-4, IL-5	↔	↔		[27]
	SP600125	Mouse	↓	↓	ND	ND	↓	↓ ASM cell proliferation	[58]

AHR, airway hyperresponsiveness; ASM, airway smooth muscle; DN PI3K-TAT, dominant negative PI3K-TAT; ECP, eosinophil cationic protein; EPO, eosinophil peroxidase; Itk, inducible T cell kinase; ND, no data; ODN, oligodeoxynucleotide; Syk, spleen tyrosine kinase.

antigen-specific IgE and airway hyperresponsiveness were significantly suppressed in a mouse model of asthma [44]. GATA3, a transcription factor regulated by STAT6 and NF-κB, is implicated in Th2 development in mouse and human T cells and is upregulated in bronchial biopsy specimens from asthmatic patients [3,30]. Intranasal administration of ASOs targeted at GATA3 suppressed antigen-induced airway inflammatory cell infiltration, mucus hypersecretion, hyperresponsiveness and IL-4 production in a mouse asthma model [45]. EGFR activation is associated with airway mucus production and airway remodelling; selective inhibition of EGFR tyrosine kinase is anticipated to produce anti-inflamamtory effects in asthma. Intratracheal instillation of the EGFR inhibitor AG-1478 dose-dependently inhibited airway eosinophil infiltration, hyperresponsiveness, mucus and MUC5AC production and collagen deposition in a mouse model of asthma [46].

PI3K inhibitors

Class 1A PI3K can be directly invoked by receptor tyrosine kinases and non-receptor tyrosine kinases, whereas Class 1B PI3K is directly activated by the Gβγ subunit of the heterotrimeric G protein upon GPCR activation. A PI3K isoform-specific inhibitor is still not available. LY294002 and wortmanin are the two commonly used PI3K inhibitors but they do suffer from some non-specific activities. Two recent reports [47••,48] revealed anti-inflamamtory effects of intratracheally administered LY294002 in mouse asthma models by suppressing antigen-induced airway inflammatory cell infiltration, production of IL-4, IL-5, IL-13, eosinophil cationic protein and eotaxin in BAL fluid, goblet cell hyperplasia, and airway hyperresponsiveness. These findings are further strengthened by similar observations using a dominant-negative mutant of the Class 1A PI3K in a mouse model of asthma [49].

MAPK inhibitors

MAPK is the converging point of many upstream signaling pathways initiated by receptor tyrosine kinases, immunoreceptors, cytokine receptors and GPCRs. MAPK pathways control many downstream signaling molecules and transcription factors critical for gene regulation. As such, inhibition of MAPK signaling pathways is expected to produce anti-inflammatory effects in asthma. A recent report by Duan *et al.* [50••] showed that U0126, a potent and selective MEK1/2 inhibitor through ablation of ERK1/2 activation, significantly blocked antigen-induced airway inflammatory cell infiltration, levels of IL-4, IL-5, IL-13 and eotaxin in BAL fluid, serum antigen-specific IgE levels, mucus production, VCAM-1 expression and airway hyperresponsiveness in a dose-dependent manner in a mouse asthma model. p38 MAPK has been a major therapeutic target for rheumatoid arthritis, as earlier studies revealed a role for p38 MAPK in the

regulation of TNF-α translation [24]. Recently, two studies examined the potential anti-inflammatory effects of p38 MAPK inhibition in animal models of asthma. Oral SB239063, a potent and selective p38 MAPK inhibitor, significantly inhibited antigen- and leukotriene D4-induced eosinophil infiltration in both mouse and guinea-pig models of asthma. In addition, SB239063 promoted apoptosis of eosinophils isolated from guinea-pig BAL fluid [51]. Using respirable isoform-selective p38α MAPK ASOs, Duan *et al.* [52••] demonstrated significant beneficial effects in a mouse asthma model through reduction of antigen-induced airway inflammatory cell infiltration, levels of IL-4, IL-5 and IL-13, mucus hypersecretion and hyperresponsiveness. Targeting JNK using the selective inhibitor SP600125 has also revealed anti-inflammatory effects in a rat model of asthma by inhibiting antigen-induced airway inflammatory cell infiltration (see also Update) [27••].

Conclusions

Substantial effort has been made to design drugs that target the tyrosine kinase signaling cascades for a variety of diseases including allergic inflammation [53–57]. Only a few of these inhibitors have been tested in *in vivo* animal models of asthma, and nearly all of these studies are at the early pre-clinical stage and require follow-up studies involving pharmacokinetic and toxicological analyses. As some of these signaling molecules in the tyrosine kinase signaling cascade are expressed ubiquitously, it is paramount to investigate potential side effects associated with the use of inhibitors targeting this signaling cascade. To circumvent systemic exposure to these signaling inhibitors, topical application (e.g. using an aerosol device for asthma therapy) is preferred to attain high local drug concentrations and activities. Intratracheal ASOs targeted at several critical signaling enzymes have shown beneficial effects in allergic airway inflammation. Those findings support a novel approach using topical ASO administration for inflammatory lung diseases. Tyrosine kinase signaling cascades represent an attractive target pathway for anti-inflammatory therapy for asthma, and target-specific inhibition of the tyrosine kinase signaling cascades might offer beneficial therapeutic effects in allergic airway inflammation.

Update

Recent work has demonstrated that SP600125 is able to suppress the antigen-induced increase in eosinophil and lymphocyte numbers in BAL fluid, eosinophil infiltration in bronchial submucosa, goblet cell and airway smooth muscle cell hyperplasia, and airway hyperresponsiveness in a chronic mouse asthma model [58••].

Acknowledgements

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Busse WW, Lemanske RF: **Asthma.** *N Engl J Med* 2001, **344**:350-362.
2. Cohn L, Elias JA, Chupp GL: **Asthma: mechanisms of disease persistence and progression.** *Annu Rev Immunol* 2004, **22**:789-815.
3. Larche M, Robinson DS, Kay AB: **The role of T lymphocytes in the pathogenesis of asthma.** *J Allergy Clin Immunol* 2003, **111**:450-463.
4. Wills-Karp M: **Interleukin-13 in asthma pathogenesis.** *Immunol Rev* 2004, **202**:175-190.
5. Cho JY, Miller M, Baek KJ, Han JW, Nayar J, Lee SY, McElwain K, McElwain S, Friedman S, Brodie DH: **Inhibition of airway remodeling in IL-5-deficient mice.** *J Clin Invest* 2004, **113**:551-560.
6. Reed CE, Kita H: **The role of protease activation of inflammation in allergic respiratory diseases.** *J Allergy Clin Immunol* 2004, **114**:997-1008.
7. Lukacs NW, Miller AL, Hogaboam CM: **Chemokine receptors in asthma: searching for the correct immune targets.** *J Immunol* 2003, **171**:11-15.
8. Amishima M, Munakata M, Nasuhara Y, Sato A, Takashi T, Homma Y, Kawakami Y: **Expression of epidermal growth factor and epidermal growth factor receptor immunoreactivity in the asthmatic human airway.** *Am J Respir Crit Care Med* 1998, **157**:1907-1912.
9. Hirst SJ, Martin JG, Bonacci JV, Chan V, Fixman ED, Hamid QA, Herszberg B, Lavoie JP, McVicker CG, Moir LM *et al.*: **Proliferative aspects of airway smooth muscle.** *J Allergy Clin Immunol* 2004, **114**:S2-S17.
10. Burgel PR, Nadel JA: **Role of epidermal growth factor receptor activation in epithelial cell repair and mucin production in airway epithelium.** *Thorax* 2004, **59**:992-996.
11. Gorska MM, Alam R: **Signaling molecules as therapeutic targets in allergic diseases.** *J Allergy Clin Immunol* 2003, **112**:241-250.
12. Kawakami T, Galli SJ: **Regulation of mast-cell and basophil function and survival by IgE.** *Nat Rev Immunol* 2002, **2**:773-786.
13. Nel AE: **T-cell activation through the antigen receptor: Part 1: signaling components, signaling pathways, and signal integration at the T-cell antigen receptor synapse.** *J Allergy Clin Immunol* 2002, **109**:758-770.
14. Dal Porto JM, Gauld SB, Merrell KT, Mills D, Pugh-Bernard AE, Cambier J: **B cell antigen receptor signaling 101.** *Mol Immunol* 2004, **41**:599-613.
15. Pitcher LA, van Oers NS: **T-cell receptor signal transmission: who gives an ITAM?** *Trends Immunol* 2003, **24**:554-560.
16. Miller AT, Berg LJ: **New insight into the regulation and functions of Tec family tyrosine kinase in the immune system.** *Curr Opin Immunol* 2002, **14**:331-340.
17. Li ZW, Rickert RC, Karin M: **Genetic dissection of antigen receptor induced-NF-κB activation.** *Mol Immunol* 2004, **41**:701-714.
18. Gagliardo R, Chenez P, Mathieu M, Bruno A, Costanzo G, Gougat C, Vachier I, Bousquet J, Bonsignore G, Vignola AM: **Persistent activation of nuclear factor-κB signaling pathway in severe uncontrolled asthma.** *Am J Respir Crit Care Med* 2003, **168**:1190-1198.
19. Poynter ME, Cloots R, van Woerkom T, Butnor KJ, Vacek P, Taatjes DJ, Irvin CG, Janssen-Heininger YMW: **NF-κB activation in airways modulates allergic inflammation but not hyperresponsiveness.** *J Immunol* 2004, **173**:7003-7009.

20. Okkenhaug K, Bilancio A, Farjot G, Priddle H, Sancho S, Peskett E, Pearce W, Meek SE, Salpekar A, Waterfield MD *et al.*: **Impaired B and T cell antigen receptor signaling in p110δ PI 3-kinase mutant mice.** *Science* 2002, **297**:1031-1034.

21. Ali K, Bilancio A, Thomas M, Pearce W, Gilfillan AM, Tkaczyk C, Kuehn N, Gray A, Giddings J, Peskett E *et al.*: **Essential role for the p110δ phosphoinositide 3-kinase in the allergic response.** *Nature* 2004, **431**:1007-1011.

22. Deane JA, Fruman DA: **Phosphoinositide 3-kinase: diverse roles in immune cell activation.** *Annu Rev Immunol* 2004, **22**:563-598.

23. Del Prete A, Vermi W, Dander E, Otero K, Barberis L, Luini W, Bernasconi S, Sironi M, Santoro A, Garlanda C *et al.*: **Defective dendritic cell migration and activation of adaptive immunity in PI3Kγ-deficient mice.** *EMBO J* 2004, **23**:3505-3515.

24. Dong C, Davis RJ, Flavell RA: **MAP kinases in the immune response.** *Annu Rev Immunol* 2002, **20**:55-72.

25. Kumar A, Lnu S, Malya R, Barron D, Moore J, Corry DB, Boriek AM: **Mechanical stretch activates nuclear factor-kappaB, activator protein-1, and mitogen-activated protein kinases in lung parenchyma: implications in asthma.** *FASEB J* 2003, **17**:1800-1811.

26. Taube C, Nick JA, Siegmund B, Duez C, Takeda K, Rha YH, Park JW, Joetham A, Poch K, Dakhama A *et al.*: **Inhibition of early airway neutrophilia does not affect development of airway hyperresponsiveness.** *Am J Respir Cell Mol Biol* 2004, **30**:837-843.

27. •• Eynott PR, Xu L, Bennett BL, Noble A, Leung SY, Nath PN, Groneberg DA, Adcock IM, Chung KF: **Effect of an inhibitor of Jun N-terminal protein kinase, SP600125, in single allergen challenge in sensitized rats.** *Immunology* 2004, **112**:446-453.
The first detailed study using a JNK-selective inhibitor in a rat model of allergic airway inflammation.

28. Schindler CW: **JAK-STAT signaling in human disease.** *J Clin Invest* 2002, **109**:1133-1137.

29. Pernis AB, Rothman PB: **JAK-STAT signaling in asthma.** *J Clin Invest* 2002, **109**:1279-1283.

30. Zhu J, Min B, Hu-Li J, Watson CJ, Grinberg A, Wang Q, Killeen N, Urban JF Jr, Guo L, Paul WE: **Conditional deletion of *Gata3* shows its essential function in T_H1-T_H2 responses.** *Nat Immunol* 2004, **5**:1157-1165.

31. Wong MM, Fish EN: **Chemokines: attractive mediators of the immune response.** *Semin Immunol* 2003, **15**:5-14.

32. Wetzker R, Bohmer FD: **Transactivation joins multiple tracks to the ERK/MAPK cascade.** *Nat Rev Mol Cell Biol* 2003, 4:651-657.

33. •• Wong WSF, Leong KP: **Tyrosine kinase inhibitors: a new approach for asthma.** *Biochim Biophys Acta* 2004, **1697**:53-69.
An extensive review of the role of receptor tyrosine kinases and non-receptor tyrosine kinases in allergic airway inflammation, and a detailed account of anti-inflammatory effects of tyrosine kinase inhibitors from *in vitro* and *in vivo* studies.

34. •• Duan W, Kuo IC, Selvarajan S, Chua KY, Bay BH, Wong WSF: **Antiinflammatory effects of genistein, a tyrosine kinase inhibitor, on a guinea pig model of asthma.** *Am J Respir Crit Care Med* 2003, **167**:185-192.
The first detailed study using the isoflavone natural product genistein, a broad-spectrum tyrosine kinase inhibitor, in a guinea-pig model of allergic airway inflammation.

35. • Wong BR, Crossbard EB, Payan DG, Masuda ES: **Targeting Syk as a treatment for allergic and autoimmune disorders.** *Expert Opin Investig Drugs* 2004, **13**:743-762.
An excellent reivew of the role of Syk tyrosine kinase in autoimmune disorders and a detailed evaluation of therapeutic effects of Syk inhibitors.

36. Stenton GR, Ulanova M, Dery R, Merani S, Kim MK, Gilchrist M, Puttagunta L, Musat-Marcu S, James D, Schreiber AD, Befus AD: **Inhibition of allergic inflammation in the airways using aerosolized antisense to Syk kinase.** *J Immunol* 2002, **169**:1028-1036.

37. Yamamoto N, Takeshita K, Schichijo M, Kokubo T, Sato M, Nakashima K, Ishimori M, Nagai H, Li YF, Yura T, Bacon KB: **The orally available spleen tyrosine kinase inhibitor 2-[7-(3,4-dimethoxyphenyl)-imidazo[1,2-c]pyrimidin-5-ylamino]-nicotinamide dihydrochloride (BAY 61-3606) blocks antigen-induced airway inflammation in rodents.** *J Pharmacol Exp Ther* 2003, **306**:1174-1181.

38. Mueller C, August A: **Attenuation of immunological symptoms of allergic asthma in mice lacking the tyrosine kianse ITK.** *J Immunol* 2004, **170**:5056-5063.

39. •• Lin TA, McIntyre KW, Das J, Liu C, O'Day KD, Penhallow B, Hung CY, Whitney GS, Shuster DJ, Yang X *et al.*: **Selective Itk inhibitors block T-cell activation and murine lung inflammation.** *Biochemistry* 2004, **43**:11056-11062.
The first detailed study using an Itk tyrosine kinase-selective inhibitor in a mouse model of allergic airway inflammation.

40. Malaviya R, Chen CL, Navara C, Malaviya R, Liu XP, Keenan M, Waurzyniak B, Uckun FM: **Treatment of allergic asthma by targeting Janus kinase 3-depedent leukotriene synthesis in mast cells with 4-(3′,5′-dibromo-4′-hydroxyphenyl)amino-6,7-dimethoxyquinazoline (WHI-P97).** *J Pharmacol Exp Ther* 2000, **295**:912-926.

41. Kudlacz E, Perry B, Sawyer P, Conklyn M, McCurdy S, Brissette W, Flanagan M, Changelian P: **The novel JAK-3 inhibitor CP-690550 is a potent immunosuppressive agent in various murine models.** *Am J Transplant* 2004, **4**:51-57.

42. •• Guarcoo D, Weizier S, Groneberg D, Joachim R, Ahrens B, Wagner AH, Hecker M, hamelmann E: **Inhibition of signal transducer and activator of transcription 1 attenuates allergen-induced airway inflammation and hyperreactivity.** *J Allergy Clin Immunol* 2004, **114**:288-295.
The first detailed study using STAT1-selective decoy oligodeoxynucleotides in a mouse model of allergic airway inflammation.

43. •• Desmet C, Gosset P, Pajak B, Cataldo D, Bentires-Alj M, Lekeux P, Bureau F: **Selective blockade of NF-κB activity in airway immune cells inhibits the effector phase of experimental asthma.** *J Immunol* 2004, **173**:5766-5775.
The first detailed study using NF-κB-selective decoy oligodeoxynucleotides in a mouse model of allergic airway inflammation.

44. Choi IW, Kim DK, Ko HM, Lee HK: **Administration of antisense phosphorothioate oligonucleotide to the p65 subunit of NF-κB inhibits established asthmatic reaction in mice.** *Int Immunopharmacol* 2004, **4**:1817-1828.

45. Finotto S, De Sanctis GT, Lehr HA, Herz U, Buerke M, Schipp M, Bartsch B, Atreya R, Schmitt E, Galle PR *et al.*: **Treatment of allergic airway inflammation and hyperresponsiveness by antisense-induced local blockade of GATA-3 expression.** *J Exp Med* 2001, **193**:1247-1260.

46. Vargaftig BB, Singer M: **Leukotrienes mediate part of OVA-induced lung effects in mice via EGFR.** *Am J Physiol Lung Cell Mol Physiol* 2003, **285**:L808-L818.

47. •• Kwak YG, Song CH, Yi HK, Hwang PH, Kim JS, Lee KS, Lee YC: **Involvement of PTEN in airway hyperresponsiveness and inflammation in bronchial asthma.** *J Clin Invest* 2003, **111**:1083-1092.
The first detailed study using PI3K-selective inhibitors LY294002 and wortmannin in a mouse model of allergic airway inflammation.

48. Duan W, Aguinaldo Datiles AMK, Leung BP, Vlahos CJ, Wong WSF: **An anti-inflammatory role for a phosphoinositide 3-kinase inhibitor LY294002 in a mouse asthma model.** *Int Immunopharmacol* 2005, **5**:495-502.

49. Myou S, Leff AR, Myo S, Boettlcher E, Tong J, Meliton AY, Liu J, Munoz NM, Zhu X: **Blockade of inflammation and airway hyperresponsiveness in immune-sensitized mice by dominant-negative phosphoinositide 3-kinase-TAT.** *J Exp Med* 2003, **198**:1573-1582.

50. •• Duan W, Chan JHP, Wong CH, Leung BP, Wong WSF: **Anti-inflammatory effects of mitogen-activated protein kinase kinase inhibitor U0126 in an asthma mouse model.** *J Immunol* 2004, **172**:7053-7059.

The first detailed study using a MEK1/2-selective inhibitor in a mouse model of allergic airway inflammation.

51. Underwood DC, Osborn RR, Kotzer CJ, Adams JL, Lee JC, Webb EF, Carpenter DC, Bochnowicz S, Thomas HC, Hay DWP, Griswold DE: **SB239063, a potent p38 MAP kinase inhbitor, reduces inflammatory cytokine production, airways eosinophil infiltration, and persistence.** *J Pharmacol Exp Ther* 2000, **293**:281-288.

52. •• Duan W, Chan JHP, McKay K, Crosby JR, Choo HH, Leung BP, Karras JG, Wong WSF: **Inhaled p38α mitogen-activated protein kinase antisense oligonucleotide attenuates asthma in mice.** *Am J Respir Crit Care Med* 2005, **171**:571-578.

The first detailed study using respirable p38α MAPK-selective ASOs in a mouse model of allergic airway inflammation.

53. Noble MEM, Endicott JA, Johnson LN: **Protein kinase inhibitors: insights into drug design form structure.** *Science* 2004, **303**:1800-1805.

54. O'Shea JJ, Pesu M, Borie DC, Changelian PS: **A new modality for immunosuppression: targeting the JAK/STAT pathway.** *Nat Rev Drug Discov* 2004, **3**:555-564.

55. Karin M, Yamamoto Y, Wang QM: **The IKK NF-κB system: a treasure trove for drug development.** *Nat Rev Drug Discov* 2004, **3**:17-26.

56. Manning AM, Davis RJ: **Targeting JNK for therapeutic benefit: from junk to gold.** *Nat Rev Drug Discov* 2003, **2**:554:565.

57. Kumar S, Boehm J, Lee JC: **P38 MAP kinases: key signaling molecules as therapeutic targets for inflammatory diseases.** *Nat Rev Drug Discov* 2003, **2**:717-726.

58. •• Nath P, Eynott P, Leung SY, Adcock IM, Bennett BL, Chung KF: **Potential role of c-Jun NH_2-terminal kinase in allergic airway inflammation and remodeling: effects of SP600125.** *Eur J Pharmacol* 2005, **506**:273-283.

This study provides new evidence of additional anti-inflammatory effects of JNK inhibition by SP600125 using a mouse asthma model.

EXHIBIT 2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Bennett, C. Frank
App. No	:	10/559401
Filed	:	September 11, 2008
For	:	OLIGONUCLEOTIDE MODULATION OF CELL ADHESION
Examiner	:	Zara, Jane J.
Art Unit	:	1635
Conf No.	:	5614

DECLARATION OF BRETT P. MONIA UNDER 37 CFR §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Brett P. Monia, based on personal knowledge or information, declare that:

1. I am Vice President of Antisense Drug Discovery at ISIS Pharmaceuticals, Inc., 1896 Rutherford Road, Carlsbad, California 92008. I received a Bachelor's degree from Stockton State College in Pomona, New Jersey, and a Doctor of Philosophy degree in Pharmacology from the University of Pennsylvania in Philadelphia, Pennsylvania. I have been employed at ISIS Pharmaceuticals, Inc. since 1989.

2. I am personally aware of results demonstrating a lack of efficacy of oligonucleotides targeted to Jun-N-terminal Kinase (JNK-1), a MAP kinase, in the treatment of airway hyperresponsiveness and/or pulmonary inflammation. A subset of these results are presented below.

3. Two different oligonucleotides targeted to mouse JNK-1 were selected for further testing based on their successful inhibitory activity in *in vitro* studies. The oligonucleotides were tested for efficacy in the treatment of airway hyperresponsiveness and pulmonary inflammation using a mouse asthma model similar to that described in Examples 30-32 of the instant application. The oligonucleotides were administered into the lungs of the mice by inhalation.

4. The endpoints assayed were airway hyperresponsiveness as determined by Penh, and inflammation as determined by eosinophil recruitment into the lung. Penh is a dimensionless parameter that is a function of total pulmonary airflow in mice during the respiratory cycle of the animal. Inhibition of Penh response to induction of airflow obstruction was done using metacholine. The lower the Penh value, the greater the airflow. A decrease in Penh is indicative of a decrease in airway hyperresponsiveness. Eosinophil recruitment into the lung are indicative of inflammation.

5. As shown in Figure A, treatment with three doses each of two different antisense oligonucleotides targeted to JNK-1 were not effective at reducing Penh at any of the doses of methacholine challenge, regardless of oligonucleotide dose. This absence of a significant effect for the 100 mg/ml dose of methacholine can be more easily seen in Figure B. There is no significant difference between treatment with oligonucleotides targeted to JNK-1 and saline vehicle.

6. As shown in Figure C, treatment with three doses of each of two different oligonucleotides does not reduce eosinophil recruitment to airways in the asthma model. There is no significant difference between treatment with oligonucleotide targeted to JNK-1 and saline vehicle.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States

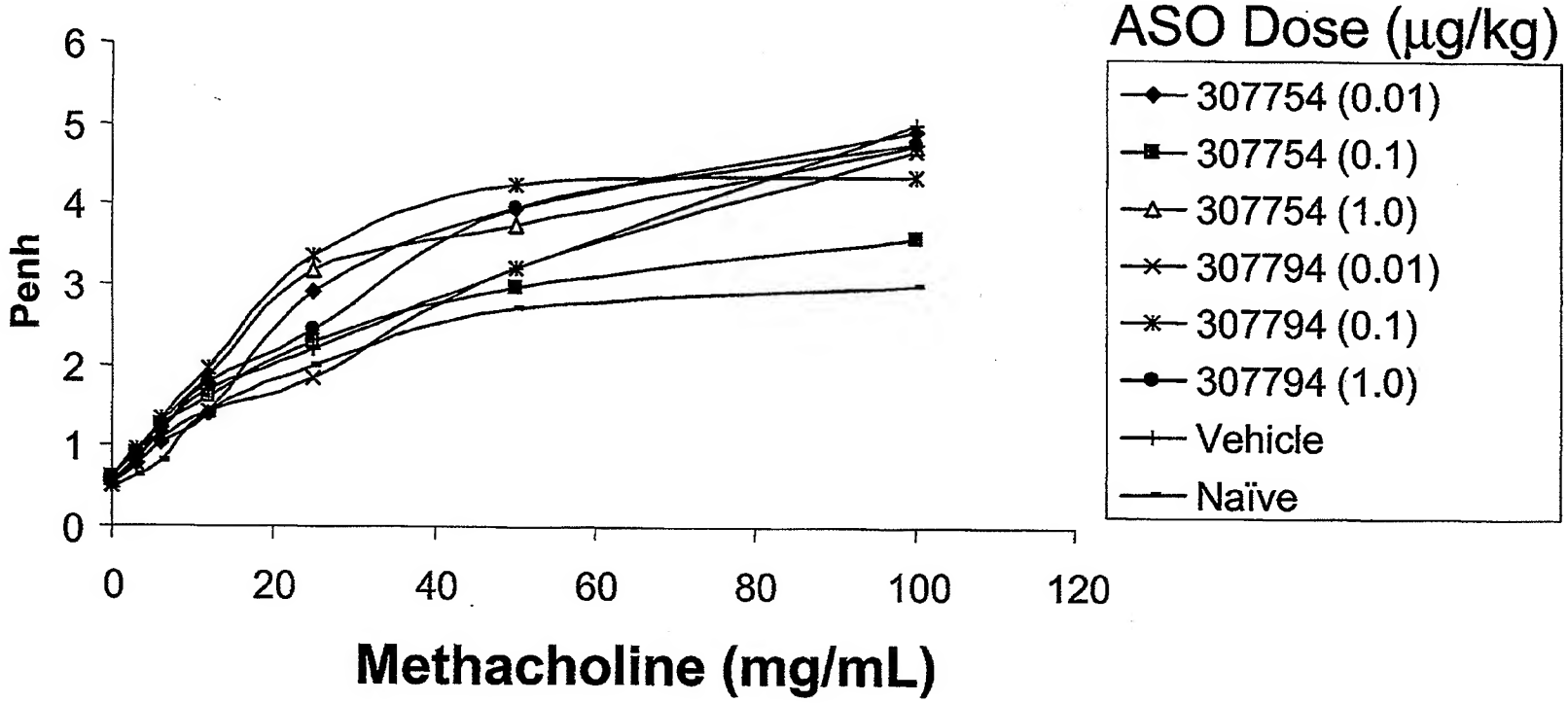
Code and that such willful statements may jeopardize the validity of the application or any patent issued thereon

Brett P. Monia, PhD

5/17/10
Date

9034736
051210

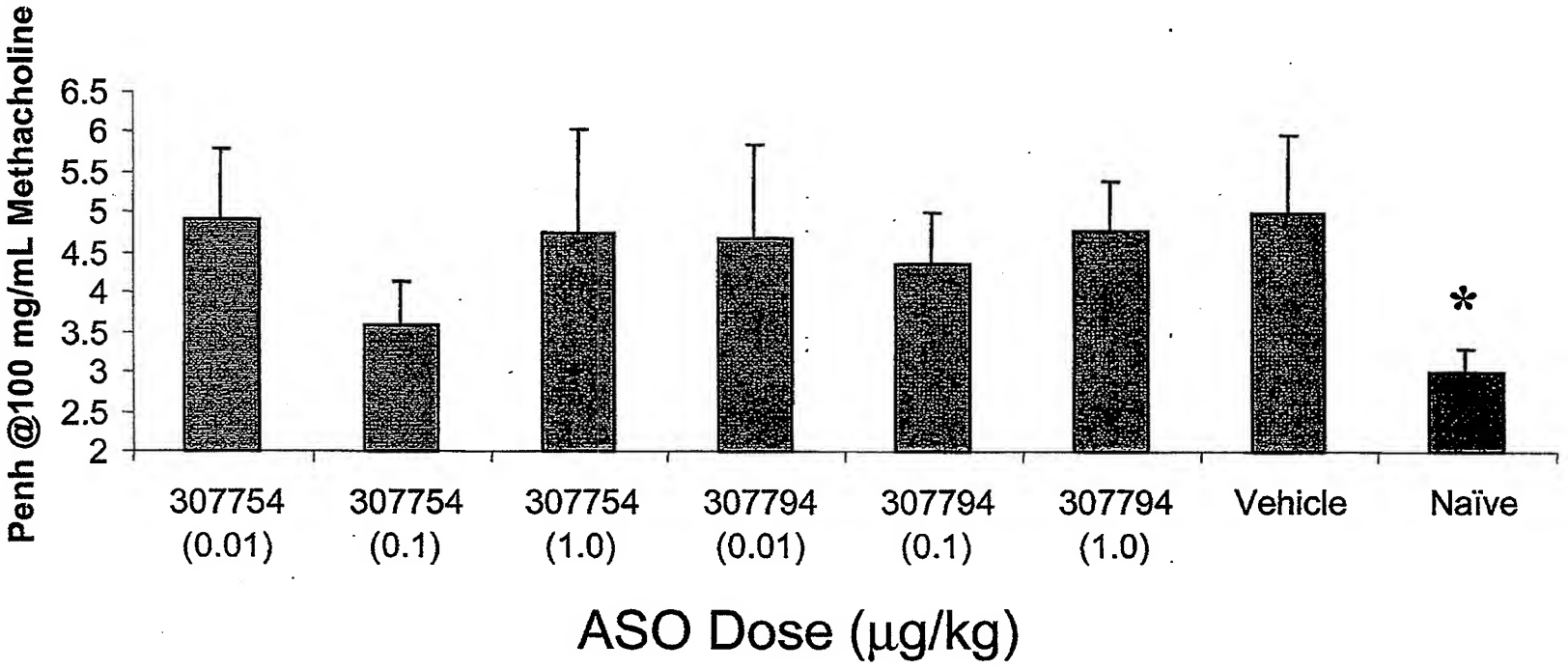
Inhalation of Jun-N-terminal Kinase (JNK-1) Antisense Oligonucleotides (ASO) Does Not Improve Airway Hyper-responsiveness (Penh) in Allergen-challenged Mice



ISIS 307754 and 307794 are chimeric 2'-O-methoxyethyl modified JNK-1 antisense oligonuceotides

Figure A

Inhalation of Jun-N-terminal Kinase (JNK-1) Antisense Oligonucleotides (ASO) Does Not Improve Airway Hyper-responsiveness (Penh) in Allergen-challenged Mice



*$p \leq 0.05$ vs. Vehicle

Figure B

Inhalation of Jun-N-terminal Kinase (JNK-1) Antisense Oligonucleotides (ASO) Does Not Reduce Eosinophil Recruitment to the Airways in Allergen-challenged Mice

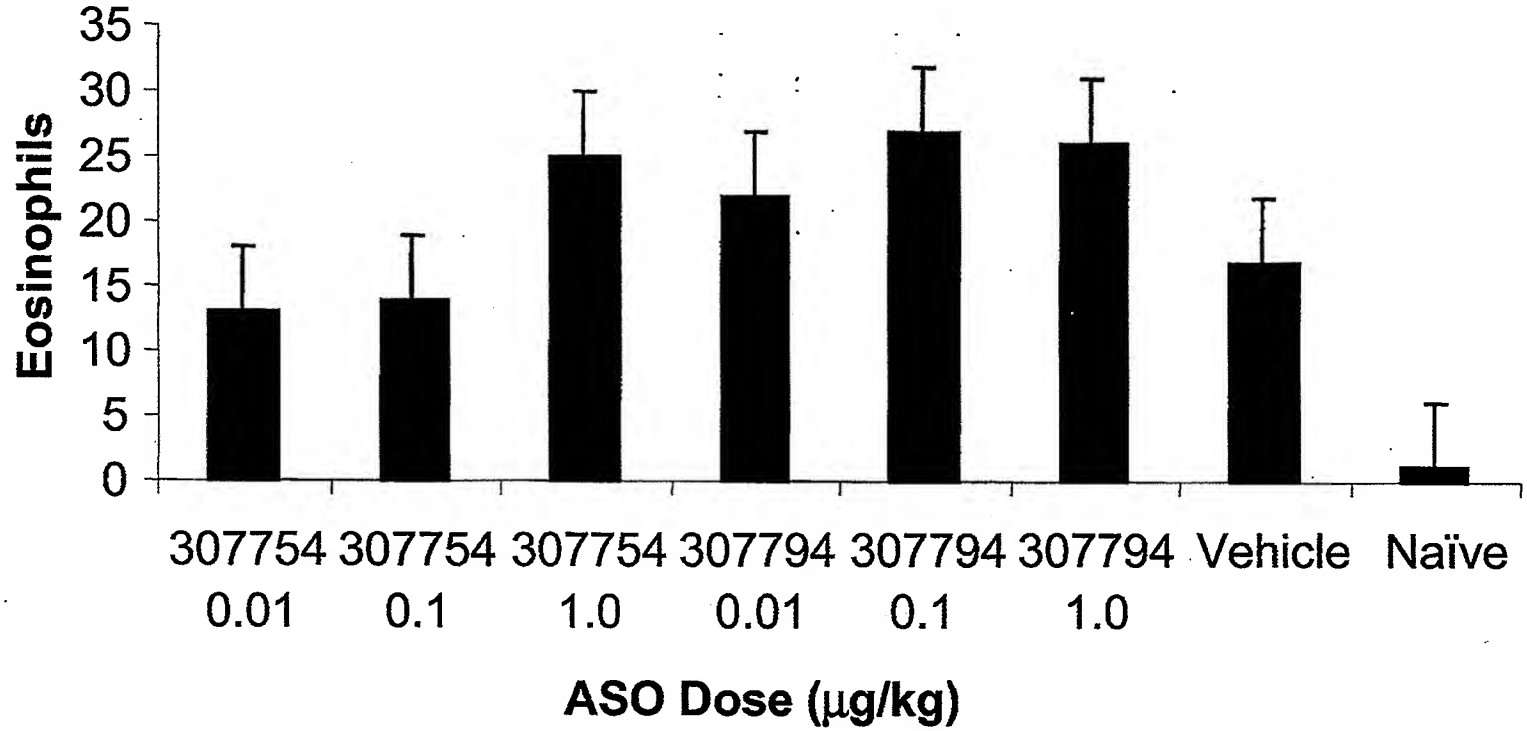


Figure C